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Use of spermatozoa from epididymus of slaughtered rams for *in vitro* fertilization of ovine oocytes

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ABSTRACT

The oocytes harvested from the ovaries obtained from a local slaughterhouse were matured *in vitro*. The oocytes reaching to metaphase II stage were 63.21%. The spermatozoa used in this study were obtained from the cauda epididymus of 2-3 testes collected from local abattoir. The washed samples were having 70-80% motility, rising up to about 90% after swimup treatment. Sperm motility was maintained throughout the capacitation period in all the 3 media. At the time of insemination above 80% of the spermatozoa exhibited progressive motility in all the 3 media. The oocyte penetration rates were 46.5, 53.4 and 54.5, respectively, for oocytes in TCM-199, Hams F10 and Hams F12 respectively. Out of the oocytes incubated further only 7,4 and 5.7% cleaved. It can be concluded that the spermatozoa collected from the epididymus of dead/slaughtered rams can be used successfully for the *in vitro* fertilization of oocytes in sheep.

Key words: Animal reproduction, *In vitro* maturation, *In vitro* fertilization, Spermatozoa, Sheep

In vitro fertilization can be used as a conservation tool for rare and endangered animal species. By collecting the gametes from animals after their death, their *in vitro* handling and *in vitro* embryo production, embryo transfer or their preservation may help in their conservation. *In vitro* maturation and *in vitro* fertilization has a very great potential for the generation of large number of embryos for research and for the application of other technologies. In bovines the capacity to produce embryos by IVM/IVF has progressed very rapidly during the past few years. However, in sheep only a limited number of offsprings have been produced using this technique. IVF can prove to be an invaluable technique not only for the supply of more number of embryos from valuable animals than what was/is possible with conventional MOET programmes, but also IVM-IVF embryos are calculated to be 5 times cheaper than the embryos from superovulated donors (Wolliams and Wilmut 1989).

Garde *et al.* (1994) have used epididymal sperms of postmortem rams up to 24 hr to determine the percentage of penetration by *in vitro* sperm penetration assays using zona free hamster eggs, and found that penetration rates did not differ much at 0-24 hr postmortem. Lot of work in the last few years has been reported on *in vitro* fertilization. Very little information is available on the utilization of gametes from dead animals. The use of dead animal ovary and epididymus for the production of viable young ones shall help to restore population of rare and endangered species. As such the present study was undertaken with the objective to study the fertilization rates of *in vitro* matured oocytes with the epididymal sperms obtained from slaughtered rams.

MATERIALS AND METHODS

Ovaries were collected from a local slaughterhouse in Srinagar city and transported to the laboratory within 3-4 hr of slaughter in Dulbecco's phosphate buffer saline (DPBS) in a thermos flask. In laboratory each ovary was freed from the surrounding tissues and overlying bursa. Each ovary was given 3 washings in DPBS and 2 washings in oocyte harvesting medium (DPBS+ 4 mg/ml BSA +50 IU/ml penicillin). The oocytes were harvested by one of the techniques, puncture, slicing or aspiration.

While harvesting the oocytes by puncture and slicing methods, the ovary was kept completely dipped in the medium in a 35 mm petri-dish. In aspiration visible follicles were aspirated using a 20-gauge hypodermic needle attached with a sterile disposable syringe having 2ml of medium. The media along with the collected oocytes was then transferred to a 35-mm petri-dish. In all the 3 techniques, the petri-dishes were kept undisturbed for 5 min, allowing the oocytes to settle down. Excess media was taken out by a syringe without

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disturbing the oocytes to settle down. Excess media was taken out by a syringe without disturbing the oocytes at the bottom of petri-dish. The petri-dish was then examined under an inverted microscope and the total number of oocytes harvested were counted. The oocytes were graded as good, fair and poor on the basis of cumulus cells and cytoplasm.

Good: Oocytes with many complete layers of cumulus cells and uniform cytoplasm.

Fair: Oocytes with thin or incomplete layers of cumulus cells and uniform cytoplasm.

Poor: Oocytes with few or no cumulus cells.

The number of good, fair and poor oocytes obtained were recorded for each ovary.

In vitro *maturation*

The good and fair oocytes were pooled and given 2 to 3 washings in maturation medium (Hams F10, or TCM-199) supplemented with 10% fetal calf serum, 0.1 IU/ml human menopausal gonadotrophin and 50 IU/ml penicillin. Ten oocytes were placed in 2 ml of maturation medium and incubated at 38.5°C under 5% with saturated humidity for 24-26 hr.

After the end of incubation, a portion of oocytes was freed from the cumulus cells by continuously pippeting in and out of a capillary, so that completely denuded oocytes were obtained. The denuded oocyte were fixed in ethanol and acetic acid solution (3:1) for 24 hr, stained with 1% aceto orecine stain and examined under a high power microscope. The maturation state of oocytes was evaluated on the basis of nuclear maturation. The oocytes were classified as germinal vesicle (GV), germinal vesicle breakdown (GVB), metaphase-I (M-I), metaphase-II (M-II) and degenerated (Dg).

Collection of spermatozoa

Sheep testes obtained from the abattoir in DPBS and taken to laboratory in a thermos flask at room temperature. They were washed with normal saline and cauda epididymus was isolated. The area below the deferent duct was identified, and was supposed to have mature sperms. This area was trimmed free of the tissues covering and washed with sterile DPBS. A prick was made on the convoluted tubules with a sterile hypodermic needle. The gushing fluid rich in sperms was collected with a micropippete and mixed with 4 ml of washing medium. Spermatozoa were collected from 2-3 epididymi of different rams mixed and used for a single insemination. Three types of media were used for sperm preparation and *in vitro* fertilization i.e. TCM-199, Hams F10 and Hams F12. The sperms were given 2 washings by centrifugation at 1500 rpm for 10 min in media supplemented with 4 mg/ml BSA and 50 IU/ml penicillin. About 100µ of loose pellet was overlaid with 2ml of fertilization medium, supplemented with 4 mg/ml BSA, 50 IU/ml penicillin and 50 IU/ml heparin and kept in CO_2 incubator (38.5°C) at an angle of 45°. The sperms were allowed to swim up for 2 hr.

In vitro *fertilization*

The matured oocytes were washed once in fertilization medium and then transferred to 2 ml of fertilization medium. The highly motile spermatozoa from the upper layers were added to the oocytes at the concentration of 1-2 x 10^6/ml approximately. The mixture of gametes was incubated for 18-22 hr. The oocytes were fixed in acid-alcohol for 24 hr, stained with 1% aceto-orcine stain and examined under high power microscope for the sperm penetration and fertilization. The sperms in perivitalline space or in vitellus, swollen sperm head in ooplasm or a male and female pronuclei were observed and recorded.

Culturing of zygotes

After coincubation of sperms and oocytes for 18-22 hr some of the oocytes were washed and transferred to 100 l droplets of culture medium pre-equilibrated under 5% CO_2. The droplets under light paraffin liquid, containing 5 oocytes/droplet, were incubated for further 44-48 hr. The cleavage rates were observed and recorded.

The data were analyzed as per Snedecor and Cochran (1967).

RESULTS AND DISCUSSION

Oocyte recovery

The oocytes were collected by puncturing the ovarian surface by an 18 gauge hypodermic needle or by slicing the ovary using a scalpel blade or by aspiration of the visible follicles. All the data were pooled and the average number of good, fair and poor oocytes recovered per ovary were 4.88±0.24, 1.91±0.15 and 1.41±0.75 respectively.

In vitro *maturation*

Oocytes recovered were matured *in vitro* and their nuclear maturation was evaluated. About 63% oocytes were in metaphase-II stage followed by metaphase-I stage (Fig. 1)

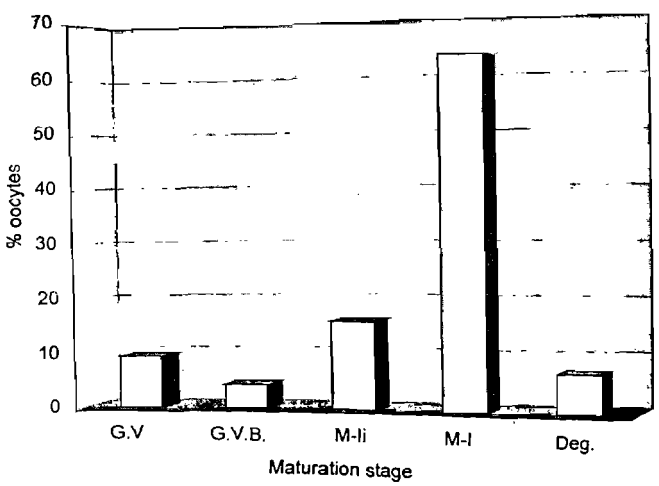

Fig. 1. *In vitro* maturation rates of ovine oocytes.

Table 1. *In vitro* fertillization of *in vitro* maturd oocytes by epididymal spermatoza in three different media

Media used	Total No.of oocytes	Oocytes (%)			
		penetrated	with polyspermy	incubated	cleaved
TCM-199	260	121.00 (46.54)	4.00 (1.54)	43.00	3.00 (7.00)
Hams-F10	148	79.00 (53.38)	1.00 (0.67)	50.00	2.00 (4.00)
Hams-F12	198	108.00 (54.54)	3.00 (1.51)	35.00	2.00 (5.7)

In vitro *fertilization*

The spermatozoa used in this study were obtained from the cauda epididymus of 2-3 testes collected from local abattoir. The washed samples were having 70-80% motility, rising up to about 90% after swimup treatment. Sperm motility was maintained throughout the capacitation period in all the 3 media. At the time of insemination above 80% of the spermatozoa exhibited progressive motility in all the 3 media. The oocyte penetration rates were 46.5, 53.4 and 54.5, respectively, for oocytes in TCM-199, Hams F10 and Hams F12 respectively. Out of the oocytes incubated further only 7,4 and 5.7% cleaved (Table 1).

In vitro fertilization can be used as a conservation tool for rare and endangered species. In such species a male is as precious as a female. The spermatozoa used in this study were collected from the cauda epididymus of slaughtered rams many hours after their death. The washed samples were having 70-80% motility and at the time of semination of oocytes above 80% spermatozoa showed progressive motility in all the 3 media, These findings are comparable to those reported by Crozet *et al.* (1987). However, they used fresh semen from rams of proven fertility. Our results are in full agreement with Garde *et al.* (1994) who used epididymal spermatozoa of postmortem rams (0-24 hr postmortem) to determine the percentage of penetration by *in vitro* sperm penetration assay using zona free hamster eggs. He found that live gametes capable of fertilization can be obtained many hours after the death of rams. Del Campo *et al.* (1994) have also used epididymal sperms for IVF in Llama successfully. Our findings demonstrate that all the 3 media can be used successfully for *in vitro* capacitation of spermatozoa and *in vitro* fertilization. Bondioli and Wright (1980) used synthetic oviductal fluid and minimum essential medium for sperm capacitation but no more than 14% of the oocytes were penetrated by the spermatozoa. Murzamadiev *et al.* (1986) used different media for sperm capacitation in sheep and found TCM-199 with sheep serum better for survival and capacitation of spermatozoa.

The fertilization rates obtained in our study were comparable to those reported by Murzamadiev *et al.* (1986) and Szollosi *et al.* (1988) who also obtained 50% and 45.7% fertilization rates, respectively, in sheep oocytes. However our results were lower than Cognie *et al.* (1991), who obtained 82.6% penetration rates. The differences might be attributed to the fact that these authors used *in vivo* matured oocytes whereas in our study *in vitro* matured oocytes were used. Fukui *et al.* (1988) obtained 43.89% fertilization rates affected by ram from which spermatozoa were taken. In our study also spermatozoa were collected from epididymus of different rams and the lower fertilization rates may be attributed to the sources of spermatozoa and ram effect. Our findings demonstrated that all the 3 media can be used for the spermatozoa preparation and *in vitro* fertilization of matured oocytes. However Hams F10 and Hams F12 proved slightly better than TCM- 199.

The polyspermy rates in our study were 1.54, 0.67 and 1.51% for TCM-199, Hams F10 and Hams F12, respectively, which are lower than reported by Cognie *et al.* (1991), who obtained 21% polyspermic oocytes and Cheng *et al.* (1986), who obtained 3% polyspermic oocytes. It indicated that the edididymal spermatozoa do not lead to more polyspermy as was thought earlier.

We compared the 3 media for culturing of presumed zygotes. The commercial media were used without any addition or alteration, except the addition of fetal calf serum. The percentage of oocytes cleaving were lower than Crozet *et al.* (1987), Shorgan *et al.* (1990), Galli and Moor (1991) and Watson *et al.* (1994), All these authors obtained a cleavage rate of about 34.81%. The lower rate of cleavage may be because of the absence of some energy substrates like sodium pyruvate, calcium lactate in the culture medium. It has been reported that first cell division requires pyruvate. This may be supplied by cumulus cells and oviductal cells *in vivo*. Oviductal pyruvate and lactate levels increases shortly after ovulation. This increase occurs only in pregnant oviducts implying acknowledgement by the maternal system that an embryo is present (Neider and Corder 1983). The other causes of lower cleavage rates can be attributed to the source of serum used. As reported by Pinlyopummintr and Bavister (1994) that different types and sources of protein supplementation in culture media can greatly influence development of mammalian embryo *in vitro*, ranging from marked stimulatory to completely inhibitory effects. In present study fetal calf serum was used, while sheep serum (Walker *et al.* 1986) or

human serum (Walker *et al.* 1988) have been reported to be superior than fetal calf serum. The presumptive zygotes were cultured in the droplets of medium, under light weight paraffin oil, which is reported to cause a low level toxicity to the embryo (Kane 1988). No attempt was made to co-culture the embryos with any cell type in this study. The co-culture of ovine embryos on monolayers of oviductal cells (Gandolfi and Moor 1987), trophoblastic vesicles (Rexroad and Powell 1988) and other reproductive tract cells (Rexroad and Powell 1993) are reported to increase the cleavage rates and number of embryos blastocyst stage.

It can be concluded that spermatozoa can be obtained from the epididymus of slaughtered/dead animals many hours after their death. These spermatozoa from valuable animals can be used for fertilization or can be preserved for future use.

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